

Production of High-Titer Helper-Free Retroviruses by Transient Transfection

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Notes:

Production of high-titer helper-free retroviruses by transient transfection

(retroviral packaging cells/gene therapy)

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ABSTRACT The generation of high-titer, helper-free retroviruses by transient transfection has been achieved by using the highly transfectable 293T cell line into which are stably introduced constructs that express retroviral packaging functions. The resulting ecotropic virus packaging cell line BOSC 23 produces infectious retrovirus at $>10^6$ infectious units/ml of supernatant within 72 hr after CaPO_4 -mediated transfection. A stringent assay for replication-competent virus showed that no helper virus was present. The system can produce high titers of retroviral vectors expressing genes that are extremely difficult to propagate at high titer in stable producer lines. This method should facilitate and extend the use of helper-free retroviral gene transfer, as well as be useful for gene therapy.

Present methods for creating high-titer, helper-free retroviral stocks employ the creation of a stable producer cell line that expresses the retroviral vector (1). Producer lines are made by introducing the retroviral vector into a packaging cell line, which synthesizes all the proteins required for viral assembly. It is then necessary to screen many clones for one clone that expresses the retroviral vector at high titer, because virus production by the original pool of transfected clones is low. Selection takes at least 1 month, and prolonged high expression of the retroviral mRNA may slow the growth of the producer line, allowing outgrowth of poorly producing variants during prolonged cultivation.

To circumvent these problems, a strategy was devised that uses transient transfection of the retroviral packaging cell line BOSC 23 to produce high-titer, helper-free infectious retroviruses. The BOSC 23 producer cell line (derived from the Ad5-transformed human embryonic kidney 293 cell line) is more transfectable than the currently employed packaging cell lines. By using BOSC 23 packaging cells and optimizing the CaPO_4 transfection procedure, we have obtained retroviral titers in excess of 10^7 infectious particles per milliliter of supernatant within 48–72 hr after transfection. The retroviruses produced are helper-free and can infect early hematopoietic progenitors. Moreover, titers in excess of 10^6 have routinely been attained by using retroviral vectors expressing genes that appear toxic to stable producer lines.

MATERIALS AND METHODS

Cell Lines and Plasmids. 293T cells (2), originally referred to as 293tsA1609neo, were obtained from S. Haase (Stanford University) and grown in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal calf serum (FCS). Drug selections in transfected 293T cells were done at 400 μg of hygromycin (Calbiochem) per ml for hygromycin resistance and 50 μg of mycophenolic acid (Sigma) per ml for guanine phosphoribosyltransferase (gpt) resistance. The following

cell lines, all grown in Dulbecco's modified Eagle's medium containing 10% (vol/vol) calf serum were used: NIH 3T3, BAG (3), N54 (4), and 210W (5).

The following plasmids, all grown in *Escherichia coli* DH5 α , were used: pBND 8 [D. Turner and C. Cepko, personal communication; a retroviral vector derived from the BAG vector (3) in which β -galactosidase (β -gal) expression is directed by the viral promoter in the long terminal repeat and the neomycin resistance cassette is deleted], pCRIPenv⁻ (6), pCRIPgag-2 (6), MFG-lacZ (7), MFG-tPA [similar to MFG-lacZ, but expressing the human tissue plasminogen activator gene in place of β -gal (7)], pZAP (8), pSV2Hgm (9), pGPT2E (10), pGD (11), pGD^{v-abl} (12), and pGD210^{bcr/abl} (11).

Enzymatic Assays and Nucleic Acid Procedures. Staining for β -gal activity in intact cells and spleen was performed as described (13). Reverse transcriptase (RT) activity was assayed in the culture medium of exponentially growing cells as described by Goff *et al.* (14). The *in vitro* abl kinase assays were performed as described by Konopka *et al.* (15) using anti-pEX4 as the immunoprecipitating antibody. Isolation of DNA and blot analysis was performed by standard procedures (16).

Transfections, Infections, and Determination of Viral Titers. Except where noted, all transfections were done on 60-mm dishes where $1.5\text{--}2.5 \times 10^6$ 293T cells (or derivatives) were plated in 4 ml of 10% FCS the night prior to transfection. Where indicated, chloroquine was added to this medium to a final concentration of 25 μM ≈ 5 min prior to adding the DNA/Hepes buffered saline solution. Either 250 or 500 μl of Hepes buffered saline solution (pH 7.05) was added to an equal volume of DNA/ CaCl_2 solution by bubbling, and the resulting solution was immediately added to the above medium (17). The cells were then returned to the 37°C incubator (5% CO_2) for 24 hr. Subsequently, the medium was changed to 3 ml of fresh 10% FCS, and 24–48 hr later, the medium was removed and either filtered through a 0.45- μm filter or centrifuged at $500 \times g$ for 5 min in a Sorvall RT6000B centrifuge. In experiments with chloroquine, the medium was changed to 10% FCS at 10 hr posttransfection and changed a second time at 24 hr posttransfection. Infections were performed as described (6). Viral titer was determined as the average number of blue (β -gal-producing) cells per 10–25 high power fields (40,000–100,000 total cells) multiplied by a factor to account for magnification, plate size, and dilution of the infectious stock. When fluorescence-activated cell sorting (FACS) analysis was performed, the percentage of positive cells was multiplied by the total number of cells on the dish. G418 selection was performed as above except that at 48 hr postinfection the cells were split 1:10 into selective

Abbreviations: β -gal, β -galactosidase; G418^R, G418 resistance; RT, reverse transcriptase; FCS, fetal calf serum; FACS, fluorescence-activated cell sorting; SV40, simian virus 40; gpt, guanine phosphoribosyltransferase.

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medium and refed every 3 days, and colonies were counted 12 days later. In determining the G418-resistance (G418^R) titer, the number of colonies was divided by 4 to account for two cell doublings. *v-abl* focus-forming activity was assayed by infecting NIH 3T3 cells with dilutions of BOSC 23-produced *v-abl* retroviral supernatants as described (18). Transformed colonies were counted 10 days after infection.

Helper Virus Assay. One milliliter of supernatant from transfected BOSC 23 cells was used to infect BAG cells as described above, and the cells were split 1:10 every 3 or 4 days. To determine the titer of the virus used to infect the BAG cells, 1 ml of the viral supernatant from the transfected BOSC 23 cells was used in parallel to infect 3T3 cells, which were assayed for β -gal or G418^R. When passages 3, 5, and 10 of the BAG-infected cells had reached $\approx 50\%$ confluence, the medium was changed, and 24 hr later, 3 ml of this supernatant was filtered (0.45 μ M) and used to infect 3T3 cells, which were stained for β -gal 48 hr later. To test for the generation of helper virus after cotransfection of the plasmids encoding the packaging constructs, 5 μ g of pCRIPenv⁻, pCRIPgag-2, and pBND 8 were cotransfected into 293T/17 cells, and 1 ml of viral supernatant was used to infect the BAG cells. The BAG cells were treated as above except that β -gal staining was performed on passage 3 cells only.

Bone Marrow Transplantation. Bone marrow transplant was performed as described (11). Recipients were 6- to 8-week-old female BALB/c mice, and 6- to 8-week-old male BALB/c mice treated with 2 mg of 5-fluorouracil 5 days prior to bone marrow harvest were donors. Cocultivation was done in WEHI-3B-conditioned medium with BOSC 23 cells that had been transfected with 6 μ g of MFG-lacZ prior to cocultivation. Nonadherent cells were removed 48 hr later and injected into the lateral tail vein of recipient mice that had received 900 cGy (two doses separated by 3 hr) at cell numbers ranging from 20,000 to 100,000 per mouse. Surviving mice were sacrificed at day 13, and the spleens were analyzed.

RESULTS

Production of high-titer retroviral stocks by transient transfection appears to be limited by the transfectability of the producer cells, the type of expression construct, and the copy number of the introduced construct. Previous studies showed that the Ad5-transformed embryonic kidney cell line 293 (19) is more transfectable than NIH 3T3 cells, the cell line from which most producer cell lines have been derived (G.P.N., unpublished data). As a result, it seemed that transient transfection of a packaging line based on 293 cells might produce high-titer retroviral stocks. To maximize our chances of transiently obtaining such stocks, we built our system from the 293T line, a 293 subline that contains the simian virus 40 (SV40) large tumor antigen (2). Although expression of SV40 large tumor antigen may increase the replication of vectors containing the SV40 origin of replication, this aspect of the 293T line was not investigated in the current studies.

Selection of a High-Producer Subline. We initially investigated whether it was possible to obtain high-titer retroviral stocks by transient transfection of 293T cells in the presence of helper virus. As a first step, the 293T cell line was cloned by limiting dilution to ensure that the starting population was uniform. Fourteen independent clones were screened by transfecting a replication-defective retroviral construct containing the *lacZ* gene (pBND 8; D. Turner and C. Cepko, personal communication). Cells from each clone were histochemically stained to detect the *lacZ* gene product. Greater than 50% of the cells from 9 clones and from the parental population expressed β -gal at 48 hr posttransfection, whereas

<25% of cells from the 5 remaining clones stained positive (W.S.P., unpublished data).

To determine which clone was capable of producing the highest titer infectious retroviral stock, seven of the nine highly transfectable clones were cotransfected with equimolar amounts (3 μ g) of pBND 8 and a clone of replication-competent Moloney murine leukemia virus [pZAP (8)]. After infection of NIH 3T3 cells and staining for β -gal activity, three clones gave the highest titer of $\approx 1.5 \times 10^6$. One of these clones (subsequently referred to as 293T/17) was chosen for further analysis. In subsequent experiments, we were able to obtain a retroviral titer of 4.6×10^6 after transfecting 2×10^6 293T/17 cells with 7.5 μ g of both pBND 8 and pZAP (Table 1).

Creation of the BOSC 23 Producer Line. To create a helper-free producer cell line, two plasmids, pCRIPenv⁻ and pCRIPgag-2, that encode the Moloney packaging functions were sequentially transfected into 293/17 cells (6) (Fig. 1). pCRIPenv⁻ contains a mutation in the envelope region and expresses both gag and pol products (6). pCRIPgag-2 contains mutations in the gag region and expresses only the ecotropic envelope (6). In addition, both plasmids were deleted for the packaging site, and the 3' long-terminal repeat was replaced by an SV40 poly(A) site (6). With these multiple changes, more than two crossover events are required for the generation of helper virus, even with the use of vectors containing packaging sites extending into gag, such as MFG (6). To create the helper-free packaging cell line, it was decided to introduce the pCRIPenv⁻ and pCRIPgag-2 constructs one after the other, rather than simultaneously, to decrease the risk of recombination and production of replication-competent virus (6).

Table 1. Retroviral production from BOSC 23 and progenitor cells after transfection

Cell line (passage no.)	Construct	CQN*	Titer†	Help‡
293T/17	7.5 μ g pBND	—	4.6×10^6	+
	7.5 μ g pZAP	—		
293T/17	5 μ g pBND	—	1.1×10^5	+
	5 μ g pCRIPgag-2	—		
	5 μ g pCRIPenv ⁻	—		
ANJOU 65	3.5 μ g pBND	—	0	
ANJOU 65	3.5 μ g pBND	—	1.5×10^5	
	3.5 μ g pCRIPgag-2	—		
BARTLETT 96 (2)	3 μ g pBND	—	1.6×10^6	
BARTLETT 96 (8)	3 μ g pBND	—	6×10^5	
BOSC 23 (2)	3 μ g MFG-lacZ	—	2.9×10^6	
BOSC 23 (6)	3 μ g MFG-lacZ	+	8.6×10^6	
BOSC 23 (12)§	6 μ g MFG-lacZ¶	+¶	1.5×10^7	
BOSC 23 (12)	3 μ g MFG-lacZ	—	6.5×10^6	
BOSC 23 (6)	3 μ g pGD	+	$3.9 \times 10^{6**}$	—
BOSC 23 (6)	3 μ g pGD ^{v-abl}	+	$2.9 \times 10^{6**}$	—
BOSC 23 (6)	3 μ g pGD210 ^{bcr/abl}	+	$1.1 \times 10^{6**}$	—
BOSC 23 (6)	3 μ g MFG-tPA††	+		—

*Presence (+) or absence (—) of 25 μ M chloroquine as described in the text.

†Infectious titer per milliliter (β -gal-positive cells).

‡Presence (+) or absence (—) of replication-competent virus as assayed in BAG cells (see *Materials and Methods*).

§Sixty percent of the cells were transfected as assayed by FACS (see *Materials and Methods*).

¶Volume of all transfection reagents was doubled.

||Thirty percent of the cells were transfected as assayed by FACS.

**Titers determined by neomycin resistance.

††Not titered, but MFG-lacZ transfected in parallel had a titer of 7×10^6 /ml.

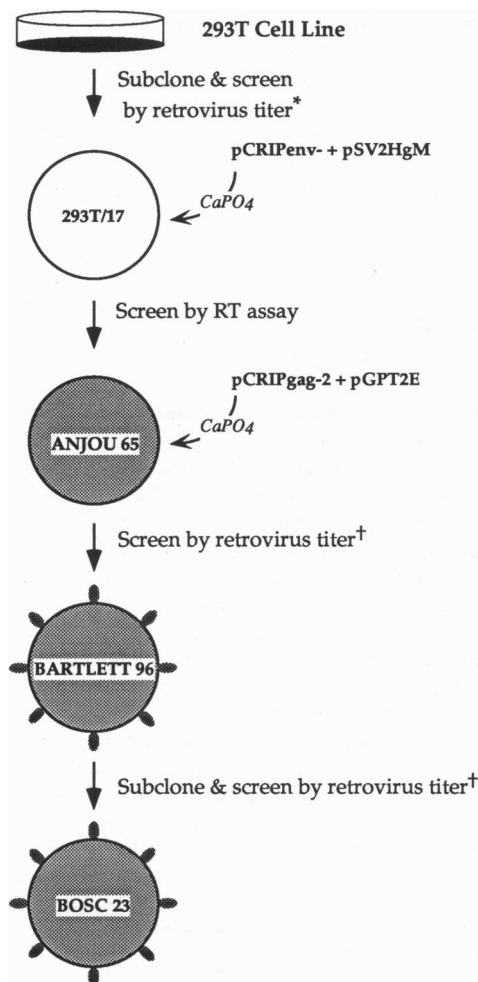


FIG. 1. Flow diagram outlining the creation of the BOSC 23 packaging cell line. Details are given in the text. *, Retrovirus titer determined after transfecting 293T clones with a *lacZ* retrovirus and helper virus; CaPO_4 , calcium phosphate-mediated transfection; †, retrovirus titer determined after transfecting indicated cell line with *lacZ* retrovirus only; shaded cells, cells stably expressing the gag-pol products of the pCRIPenv⁻ plasmid; ●, cells stably expressing the ecotropic envelope expressed from the pCRIPgag-2 plasmid.

The first step in creating the helper-free line was to create a cell line expressing high levels of gag-pol. Six micrograms of *Ase* I-linearized pCRIPenv⁻ plasmid was transfected into 293T/17 cells together with 3 μg of a plasmid encoding hygromycin as a selectable marker. Individual colonies, grown in hygromycin selection medium, were picked and screened for RT activity (14). Of 175 clones assayed, four clones produced similar or greater amounts of RT than CRE cells, a 3T3-derived producer cell line made by transfecting the pCRIPenv⁻ and pCRIPgag-2 constructs (6). The best gag-pol producing cell line, no. 65 (subsequently referred to as ANJOU 65), produced RT at a level 10 times that of CRE and 1/10th that of wild type Moloney virus-infected cells (Fig. 2). The RT activity of ANJOU 65 remained stable for at least 15 passages, even when the cells were grown without hygromycin selection. Transient cotransfection of ANJOU 65 cells with pBND 8 and the envelope-expressing construct, pCRIPgag-2, gave a retroviral titer of 1.5×10^5 (Table 1).

The next step was to transfect the ecotropic envelope construct and select a cell line producing the highest titer of infectious retrovirus. ANJOU 65 cells were transfected with 10 μg of *Ase* I-linearized pCRIPgag-2 and 5 μg of pGPT2E, a plasmid encoding the selectable marker gene for gpt resistance (10). The cells were initially grown in gpt selection

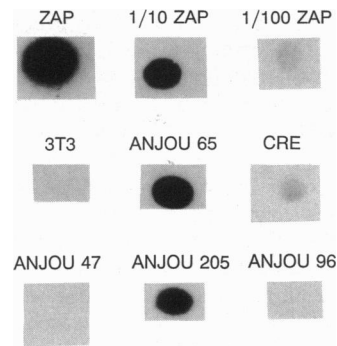


FIG. 2. RT assays of supernatants from clones of ANJOU cells transfected with pCRIPenv⁻. Individual clones resistant to hygromycin were isolated and grown, and the presence of RT activity in the medium was assayed by incubating 10 μl of cell supernatant with 50 μl of a reaction cocktail containing 50 mM Tris-HCl (pH 8.3), 20 mM dithiothreitol, 0.6 mM MnCl_2 , 60 mM NaCl, 0.05% Nonidet P-40, 5 μg of oligodeoxythymidylic acid per ml, 10 μg of polyribonucleic acid per ml, and 10 μM [α - ^{32}P]dTTP (specific activity, 1 Ci/mmol; 1 Ci = 37 GBq). The reaction was incubated for 1 hr at 37°C, spotted onto DE-81 paper (Whatman), washed, and autoradiographed (14). Positive controls are supernatant from 293T/17 cells transfected with pZAP [wild-type Moloney virus, (8)] and supernatant from CRE cells (6). The supernatants from the pZAP-transfected cells were diluted 1:10 and 1:100 as indicated. NIH 3T3 supernatant is included as a negative control.

medium (20), but once individual clones reached sufficient density to be grown on 10-cm plates, selection was removed and they were screened as described in *Materials and Methods*. Of 70 clones screened, only one clone, no. 96 (subsequently referred to as BARTLETT 96), produced *lacZ* retrovirus with a titer in excess of $10^6/\text{ml}$ following transfection with pBND 8 (Table 1).

Because BARTLETT 96 and two subcloned lines all demonstrated a decrease in the ability to package retrovirus over time when grown without gpt selection (Table 1 and W.S.P., unpublished data), it appeared that gpt selection might be necessary for maintenance of the high-titer phenotype of this subline. To test this hypothesis, passage 2 cells of the BARTLETT 96 line were thawed and cloned by limiting dilution, either in the presence or absence of gpt selection. For these experiments, MFG-*lacZ*, a β -gal-expressing retroviral vector containing an extended retrovirus RNA packaging site was used in the transfection assay (7). After transfection of the BARTLETT 96 cells, this vector produced infectious retroviral stocks with titers \approx 2-fold higher than pBND 8, which does not contain the extended packaging site (W.S.P., unpublished data). Twenty-two clones maintained in gpt selection medium were analyzed, and 7 of these clones had titers greater than $10^6/\text{ml}$. Of these 7 clones, the clone producing the highest titer, BOSC 23, gave a titer of $2.9 \times 10^6/\text{ml}$ (Table 1). BOSC 23, used in all subsequent experiments, was maintained in gpt selective medium, and its viral titer remained stable for at least 14 passages (Table 1).

Chloroquine Treatment of BOSC 23 Cells Doubles the Infectious Titer. To maximize the infectious titer, several modifications were made to the CaPO_4 transfection protocol. Growing the BOSC 23 line in 10% FCS with 25 μM chloroquine for the initial 10 hr of transfection resulted in a 2- to 3-fold increase in titer (Table 1). This is presumably due to the lysosomal neutralizing activity of the chloroquine (16). Doubling the volume of all transfection reagents also appeared to result in a slightly higher transfection efficiency (Table 1). The effects of chloroquine and reagent volume on infectious titer appeared to be the result of increasing transfection efficiency because changes in the percent of cells transfected, as measured by FACS, were nearly linear with viral titer

(Table 1; G.P.N. and W.S.P., unpublished data). Using these conditions, we have been able to transfect as many as 60% of the BOSC 23 cells, and this has resulted in retroviral stocks producing *lacZ* virus titers in the 10^7 /ml range (Table 1).

Transient Transfection of BOSC 23 Produces High-Titer *abl*-Expressing Retroviral Vectors. One reason for creating the BOSC 23 packaging line was to obtain high-titer retroviral stocks transducing genes that may be difficult to obtain at high titers from stably selected producer lines. Previous results in this laboratory have shown that it is very difficult to maintain either *v-abl* or P210^{bcr/abl} helper-free retroviral producer cell lines at titers greater than 10^5 /ml (M.L.S. and W.S.P., unpublished data). To test whether our transient system was capable of producing these recombinant retroviruses at a higher titer, either pGD^{v-abl} (12) or pGD210^{bcr/abl} (11) was transfected into BOSC 23 cells. Resulting supernatants were then titered by G418 selection for the neomycin-resistance gene also contained in the retroviral vector (11). The parental pGD vector (11), expressing only the neomycin-resistance gene, produced a viral titer of 3.9×10^6 /ml (Table 1). pGD^{v-abl} produced virus at a G418^R titer of 2.9×10^6 /ml (Table 1), and pGD210^{bcr/abl} produced virus at a G418^R titer of 1.1×10^6 /ml (Table 1). To show that the *abl* gene was transduced as efficiently as the G418^R marker, we infected NIH 3T3 cells with dilutions of BOSC 23-produced pGD^{v-abl} retrovirus. This gave a titer of 2.1×10^6 transformed foci per ml (Fig. 3A), consistent with the titer determined by G418^R. To demonstrate that the correct *abl* protein product was made by the BOSC 23-produced retroviral stocks, *in vitro* *abl* kinase assays were performed on the infected 3T3 cells (15). The P160^{v-abl} protein immunoprecipitated from the pGD^{v-abl}-infected cells was identical in size to the P160^{v-abl} from a cell line expressing Abelson murine leukemia virus [N54 (4)] (Fig. 4A). Similarly, P210 was immunoprecipitated from pGD210^{bcr/abl}-infected 3T3 cells, and the protein was identical in size to the P210 product of the 210W cell line (5), a cell line expressing P210^{bcr/abl} (Fig. 4B).

Tests for Replication-Competent Virus. To test for the production of replication-competent virus, the BAG indicator cell line was used (3). This cell line contains an integrated β -gal provirus capable of being rescued by either helper virus or retroviral packaging functions and is able to detect infectious replication-competent retrovirus in 1 ml of supernatant from a known retroviral producer line at dilutions up to 5×10^6 /ml (W.S.P., unpublished data). BAG cells were infected with 1 ml of viral supernatant from pGD (G418 titer: 3.9×10^6 /ml), pGD^{v-abl} (G418 titer: 2.9×10^6 /ml), or MFG-tPA (not titered; however, MFG-lacZ transfected in parallel had a titer of 7×10^6 /ml). Cells were split every 2–3 days at 1:10, and 3 ml of supernatant was used to infect 3T3 cells after passages 3, 5, and 10 of the BAG-infected cells. No β -gal-

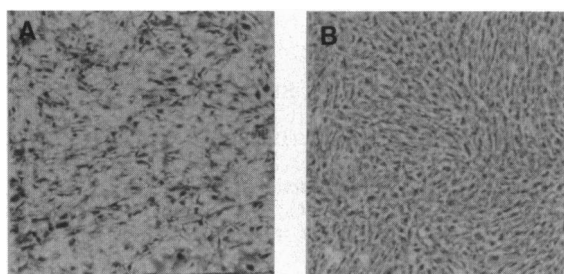


FIG. 3. Morphologic transformation of 3T3 cells by BOSC 23-produced *v-abl*. NIH 3T3 cells (5×10^5 cells plated the night before infection) were infected with 1 ml of undiluted 48-hr supernatant from either pGD^{v-abl}-transfected BOSC 23 cells (A) or pGD-transfected BOSC 23 cells (B) as described in *Materials and Methods*. G418^R titers for these stocks were 2.9×10^6 /ml (pGD^{v-abl}) and 3.9×10^6 /ml (pGD). Corresponding focus-forming titers were 2.1×10^6 /ml for the pGD^{v-abl} stock and $<10^4$ /ml for the pGD stock. Photos were taken 48 hr after infection.

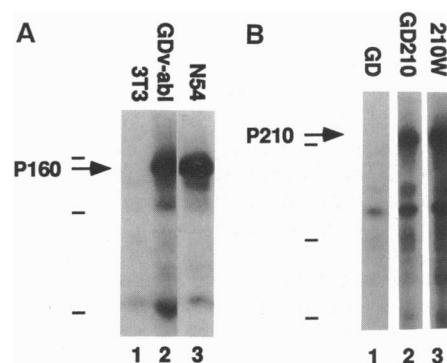


FIG. 4. *In vitro* *abl* kinase activity following infection of NIH 3T3 cells with supernatant from BOSC 23-produced P160^{v-abl} retrovirus or P210^{bcr/abl} retrovirus. The P160^{v-abl} (A) and P210^{bcr/abl} (B) proteins are indicated by arrows. Three micrograms of the retroviral vector (pGD^{v-abl}, pGD210^{bcr/abl}, or pGD) was transfected into BOSC 23 cells in the presence of 25 μ M chloroquine as described in the text. Forty-eight hours posttransfection, 1 ml of the supernatant was used to infect NIH 3T3 cells, and 48 hr later, the cells were extracted and precipitated with anti-*abl* antiserum (pEX4) as described in ref. 15. Samples were fractionated by electrophoresis through a 7.5% SDS/polyacrylamide gel, and the phosphorylated proteins were detected by autoradiography for 12 hr with an intensifying screen. Prior to the immunoprecipitations, protein concentrations were equal, except that there was approximately half as much protein in the N54 cell extract. (A) Lane 1, NIH 3T3 cells (negative control); lane 2, pGD^{v-abl}-infected NIH 3T3 cells; lane 3, N54 cells (positive control) (4). (B) Lane 1, pGD-infected NIH 3T3 cells (negative control); lane 2, pGD210^{bcr/abl}-infected NIH 3T3 cells; lane 3, 210W cells (positive control) (5). Size markers are 207, 111, and 71 kDa.

positive cells were observed upon infection of 3T3 cells with supernatants derived from cells that had undergone 9, 15, and 30 rounds of replication (Table 1). As a positive control, MFG-tPA and pZAP were cotransfected, and the supernatant was able to rescue the provirus at all of the above time points.

To test the hypothesis that simultaneous introduction of the two plasmids encoding the gag-pol and env functions would produce replication-competent virus by recombination, pCRIPenv⁻, pCRIPgag-2, and pBND 8 were cotransfected into 293T/17 cells, and 48 hr later, 1 ml of supernatant was used to infect BAG cells. The BAG cells were split 1:10 for three passages, and 1 ml of supernatant was used to infect 3T3 cells, which were stained for β -gal activity. β -gal-positive cells were present, indicating that cointroduction of these three elements results in the formation of helper virus (Table 1).

Retroviral Vectors Produced by BOSC 23 Cells Are Capable of Infecting Day 13 Colony-Forming Unit-Spleen. To show that BOSC 23 cells transfected with a retroviral vector could be used to infect hematopoietic progenitors, day 13 colony-forming unit-spleen assays (21) were carried out by using cocultivated murine bone marrow. Ficoll-banded cells (1.25×10^6) derived from mouse tibia were cocultured in WEHI-3B-conditioned medium with BOSC 23 cells that had been transfected 1 day previously with MFG-lacZ. Parallel experiments showed that the MFG-lacZ-transfected cells produced virus at a titer of 5×10^6 /ml. Forty-eight hours later, the nonadherent cells were washed off the adherent layer, and 20,000 cells were injected into each lethally irradiated recipient. The animals were sacrificed 13 days after reconstitution, and 20 distinct spleen colonies were dissected. DNA prepared from each colony was cleaved with *Xba* I (which cuts once in each retroviral long terminal repeat) and blotted to nylon membranes. Hybridization with a β -gal probe revealed the expected 4.0-kb fragment in 10 out of 15 analyzable colonies (W.S.P. and M.L.S., unpublished data).

DISCUSSION

A method for the rapid generation of high-titer stocks of helper-free retroviruses is described. By introducing constructs expressing retroviral packaging functions into a subclone of 293T cells, a retroviral packaging cell line, BOSC 23, was created. BOSC 23 cells are able to produce infectious retroviral stocks with titers as high as 10^7 infectious particles per ml within 72 hr after transfection and without selection. Retroviral stocks of high titers are necessary for efficient introduction of recombinant genetic material into rare cells, such as stem cells and infrequently dividing cells such as neurons, and to be able to infect all cells in a large target population.

A major advantage of the BOSC 23 system is the ability to produce high-titer retroviral stocks expressing genes that are detrimental to the growth of stable producer cell lines. Stable producer cell lines expressing either *v-abl* or P210^{bcr/abl} retroviral vectors showed that even when these cell lines initially expressed the retroviral constructs at high titer, the titers fell with continued propagation of the cell line (M.L.S. and W.S.P., unpublished data). Although this phenomenon is not well understood, it may be due to mechanisms relating to the degree of transformation and cellular adherence rather than a direct toxic effect of the gene product (22). This phenomenon is not limited to retroviral constructs containing members of the *abl* family because we have seen a similar phenomenon with members of the *rel* family of transcription factors (M.L.S., unpublished data). Using the BOSC 23 system, titers in excess of 10^6 /ml for both the *abl*- and *rel*-related vectors have been achieved (M.L.S., unpublished data). Because BOSC 23 cells are exposed to the retroviral products for less than 72 hr, detrimental effects of the gene product upon the producer cell line are minimized. The finding that the pGD^{v-abl} and pGD^{bcr/abl} titers are lower than those from the parental pGD vector suggests that there may still be a toxic effect; however, even with this effect, the resultant titers are $>10^6$ /ml.

The BOSC 23 system is also free of detectable replication-competent virus. This was demonstrated by the use of a stringent assay in which either packaging of the helper functions or production of helper virus itself would result in the rescue of the integrated *lacZ*-containing provirus from the BAG cells. No rescue was seen using three different retroviral constructs, including a vector containing an extended retroviral RNA packaging site (MFG-tPA), even though each BAG-infected population underwent at least 30 rounds of replication.

There have been several previous attempts to make high-titer retroviruses by transient transfection. Transient transfection of either the Psi-2 or PA317 cell lines results in titers in the 10^4 /ml range (3, 23). Recently, Landau and Littman (24) have described a system in which two retroviral vectors containing SV40 *ori* sequences and expressing the packaging functions from one vector and the gene of interest from the other vector are cotransfected into COS cells. They have obtained titers as high as 1×10^5 , which appear helper free, and are dependent on the amplification of the SV40 *ori*-containing vectors. Although only one recombination event is necessary to create replication-competent virus in their system, they do not report the presence of helper virus (24). Cotransfection of 293T/17 cells with pCRIP^{env}-, pCRIP-gag-2, and pBND, which contain the same region of homol-

ogy as in the COS-based system but requires more than two recombination events to generate helper virus, results in replication-competent virus. While we would not have anticipated the observed absence of helper virus in the COS-based system, one possible explanation is that COS cells recombine unintegrated DNA less frequently than 293 cells.

The BOSC 23 cell line should have many applications. These include testing the efficacy of different retroviral vectors and conditions, overexpressing proteins, and creating animal models of disease. The present work also suggests that an amphotropic counterpart to the BOSC 23 cell line may be useful in human gene therapy.

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